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Emulsification and antioxidation of biosurfactant extracts from Chinese medicinal herbs fermentation *in vitro*

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Much attention has been paid to biosurfactants produced using microorganisms, but little direct evidence for the development of natural biosurfactants combined with Chinese medicinal herbs are available. We investigated the emulsification and antioxidation of biosurfactant extracts from Chinese medicinal herb fermentation (BECMHF) *in vitro* and their application in water retention capacity and the skin prick and allergy test (SPAT) index for skin cells. The results showed that the water retention capacity of BECMHF was positively associated with the emulsification index. The SPAT index of 8 Chinese medicinal herbs was 0 at a 1% or 2% concentration, suggesting no sensitivity or adverse effects on the skin cells. Eight BECMHFs produced using *Alcaligenes piechaudii* CC-ESB2 exhibited antioxidant capabilities, including 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and superoxide scavenging activity, and superoxide dismutase (SOD)-like activity at a concentration of 10 mg/ml. The mechanism involved inhibitory effects on nitrite, inducible nitric oxide synthase (iNOS) expression, and reactive oxygen species (ROSs) generation. BECMHFs exhibit favorable antioxidative properties in health food and satisfactory emulsifying and moisturizing characteristics in cosmetic formulations, which have potential applications in the health food and cosmetic industries, respectively.

[**Key words:** Biosurfactant; Chinese medicinal herbs; Emulsification; Fermentation; Antioxidant]

Surfactants are a type of essential and attractive chemical that are widely used in the petroleum, pharmaceutical, cosmetic, food, pesticide, and cleaning industries. Among the surfactants, much attention has been paid to biosurfactants produced from microorganisms because of their biodegradability, high surface activity, and low toxicity. Chemical surfactants have traditionally been used to enhance the emulsification function in food and cosmetic products. However, little direct evidence for the development of natural biosurfactants combined with Chinese medicinal herbs (CMHs) is available. These potential green products are developed from a bacterial strain named *Alcaligenes piechaudii* CC-ESB2, which with higher emulsification activity, hydrophobicity and biosurfactant-producing characteristics, is a hydrocarbon-biodegrading bacterium isolated from oil-contaminated soil (1). The role of *A. piechaudii* CC-ESB2 fermented solution was reported earlier by Chen et al. (2) in enhancing the antioxidative activities of Chinese medicinal herbs including *Rhodiola rosea* and *Lonicera japonica* Thunb. Therefore, in this study *A. piechaudii* CC-ESB2 is used to ferment with CMHs including *Monascus purpureus* (M), *Spirulina platensis* (S), *L. japonica* Thunb (L), *R. rosea* (R), *Cassia nomame* (C), *Garcinia cambogia* (G), *Opuntia dillenii* (Ker), Haw (O), and *Phaseolus vulgaris* (P). Herbs are used in several products, including medicine, nutrition, flavoring, beverages, dyes, repellents, fragrances, and cosmetics (3). Numerous biologically active ingredients are found in natural herbs that are used to prevent and treat human diseases, such as cancer and cardiovascular disease. For example, several studies on *Monascus*-fermented products have determined that their biologically active metabolites can reduce cholesterol, triglycerides, and low-density lipoprotein levels in egg yolk and rodent blood (4–7). Research on CMHs that offer health benefits has proven that *S. platensis* reduces serum cholesterol and increases HDL-cholesterol in rabbits (8). Kanupriya et al. (9) suggested that the alcoholic and aqueous extracts of *R. rosea* have markedly cytoprotective and antioxidative activities. Kim et al. (10) determined that 70% (v/v) aqueous ethanol extract of 100 μg/ml of *C. nomame* exhibited 91.2% 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The biologically active ingredients of garcinol occurring in *G. cambogia* were reported to exhibit DPPH radical scavenging activity and antitumor activity (11,12). The seed coat methanol extracts, tannin fractions, and pure flavonoids of *P. vulgaris* all exhibited antioxidative activity in a fluorescence-based liposome assay (13). Additional extracts and active compounds, primarily those including compositions of essential oils, organic acids, and flavones from *L. japonica*, have been isolated and proven. They exert antiinflammatory, antiviral, antibacterial, and antioxidative effects and enhance immune response (14). Thus, the aforementioned CMHs provide substantial health benefits and warrant investigation because of their nutritional and therapeutic properties.

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Numerous oil-degrading microorganisms produce biosurfactants to enhance the use of oil substrates through increased oil solubility or dispersion. Much attention has been focused on biosurfactants that exhibit hydrophilic and lipophilic properties because of their ability to eliminate free radicals during oil degradation. In addition, they can replace chemical surfactants exhibiting nonbiodegradability and toxicity. To prevent an oxidative reaction by using artificial antioxidant chemicals, natural biosurfactants have been produced and applied in food and cosmetic research. However, they are seldom produced by microorganisms fermented using CMH fermentation (CMHF), and few studies have focused on the antioxidative effects of biosurfactants combined with CMHF. *A. piechaudii* CC-ESB2 has been reported to be a reliable oil-degrading microorganisms because of its beneficial surface-active properties, such as the cell surface hydrophobicity (82%), emulsifying activity (71% in the emulsification index), and surface tension reduction (the surface tension of the medium was reduced from 58 to 48 mN/m) of its biosurfactant (15). This study focused on potential green products developed from *A. piechaudii* CC-ESB2 using CMHF. The BECMHF were developed by investigating multifunctional properties, including antioxidation, emulsification, and moisture capacity, as well as by conducting skin irritation and allergy tests for application in the food and cosmetic industries.

MATERIALS AND METHODS

Preparation, purification and characterization of biosurfactant The *A. piechaudii* CC-ESB2 was used to produce biosurfactant in this study, which is a good diesel-oil biodegrading bacterium isolated from oil-contaminated soil. The potential biosurfactant was produced by *A. piechaudii* CC-ESB2 which was incubated in 200 ml of Bushnell and Haas (BH) medium (16) with 1.0% of soybean oil in a conical flask. After 5 days of incubation, the soybean oil layer and thalli in the conical flask were first removed. The isolation and purification of amphiphilic compounds were carefully performed referring to the procedure of Wei et al. (2005) (17) to extract the potential biosurfactant as follows: The collected culture supernatant was first centrifuged at 9000 $\times g$ for 15 min to remove *A. piechaudii* CC-ESB2 cells. The potential biosurfactants were then precipitated by acidification to pH 2.0 with 1N HCl. After centrifuged at 9000 $\times g$ for 20 min, the precipitate was extracted with ethyl acetate. The organic layer was then separated and evaporated. The biosurfactant powder was obtained through a freeze drying method using Freezer Dryer (FD-20L-6S, KINGMECH, Taiwan) from the reconcentrated liquid biosurfactant solution. The concentrated biosurfactant was dissolved in chloroform and then isolated by TLC on silica plates (Merck, Silica gel 60G F254) with chloroform/methanol/7 M NH_4OH (65:15:2, by vol.) (18), then, the obtained biosurfactant was subjected to analysis with nuclear magnetic resonance (NMR) spectrum analysis (BRUCKER AVANCE 500, Bruker, Germany) referring to the literature (19). Finally, the biosurfactant solution was prepared by adding 1.0 g of biosurfactant powder in 100 ml of deionized water (ca. concentration: 10 mg/ml) for assays of physical chemistry properties (including pH value (pH-510, EUTECH, Malaysia) and EC value (pH-510, EUTECH, Malaysia)), surfactant properties (including the reduction of surface tension (model CBVP-3; Kyowa Interface Science, Tokyo)) and critical micelle concentration (20), and emulsification property (including emulsificaton index (21) and hydrophobicity (22)).

Biosurfactant fermentation and CMHF preparation The pure strain culture of *A. piechaudii* CC-ESB2 was incubated in 200 ml of modified BH medium with 1.0% of soybean oil in a 500-ml conical flask. The modified BH medium consists of K_2HPO_4 (1 g/l), KH_2PO_4 (1 g/l), NH_4NO_3 (1 g/l), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), $CaCl_2 \cdot 2H_2O$ (0.2 g/l) and $FeCl_3$ (0.1 g/l). The bacterium was cultivated for 3 d in a shaking incubator (180 rpm) at 37°C to produce a potential biosurfactant and harvested as the inoculum for the BECMHF experiment in 1800 ml of deionized (DI) water. The BECMHF experiment was conducted in a 5-L fermenter at 30 ± 0.5°C, a pH value of 7.0 ± 0.5, 160-rpm agitation, and an aeration rate of 1 l/min of air for controlling 50–60 ppm dissolved oxygen and another BECMHF experiment was performed for 5 d involving water in oil fermentation of 8 scientific CMHs as the control, respectively. The 8 scientific CMHs were first prepared by freezing the dried matter and grinding it to a powder and then passing it through a 60-mesh sieve. Twenty grams of the frozen-dried powder of 8 scientific CMHs were first mixed separately using a stirring mixer in warm DI water (45 ± 2°C) for 5 min to availably extract the effective components of scientific Chinese medicinal herbs, then dissolved up to 1800 ml of DI water (22 ± 2°C). After 5 min, the solution was filtered through filter paper to obtain the CMH sample liquors for conducting the BECMHF experiment by adding 200 ml of inoculum for 5 d of fermentation. Finally, the 8 fermentation solutions of BECMHF were harvested by freeze-drying as frozen-dried powders and prepared by dissolving 1.0 g of dry BECMHF powders in 100 ml of DI water (22 ± 2°C), producing a final concentration of 10 mg/ml for the following experiments.

Measuring the emulsification index The emulsification ability of the BECMHF was determined by measuring the emulsification index. Briefly, a 3-ml solution of each BECMHF was added to a tube containing 2 ml of soybean oil at a ratio of 3:2. After vigorous mixing, the mixture was remained still for 24 h and the emulsification index was then determined as follows (21):

$$\text{Emulsification index } (E_{24}, \%) = [(\text{height of emulsion layer})/(\text{Total height of solution})] \times 100 \quad (1)$$

Determination of water retention capacity Ten milliliters of the 8 BECMHF solutions was separately mixed with the base formulation to produce skin moisturizers at a ratio of 1:10 (v/v). The water retention capacity of each skin moisturizer was determined according to the method described in the literature with slight modification (23). Briefly, 0.2 mL of skin moisturizer was daubed onto the skin within a 5-cm diameter in a controlled room at 25 ± 1°C and 45 ± 2% relative humidity (RH). The water retention capacity was detected on the forearm skin of 30 voluntary students aged between 18 and 20 years old. The experiment was performed after 20 min of overlay on the skin by using a moisture titrator (Karl-Fisher, Schott, Germany).

Skin prick and allergy tests SPATs were performed using the method described in the literature to estimate the safety and sensitivity of skin moisturizers (24,25). Briefly, 30 volunteers participated in this investigation. One and two percent concentrations (w/v, %) of the 8 BECMHF solutions were separately considered for the SPATs at 25 ± 2°C and under an RH condition of 60 ± 5%. The tests were performed twice daily on the forearm skin of 30 volunteer students in the morning and evening. The effect of the SPATs was estimated after 2 weeks.

Determining the antioxidative ability of BECMHF Antioxidative ability is not only an indicator of biological activity, but also a stability indicator for new products during the manufacturing and storage processes. Thus, the antioxidative abilities of BECMHFs were measured to represent their biological activities and stabilities. Assays of DPPH radical scavenging activity, superoxide scavenging activity, and superoxide dismutase (SOD)-like activity were used to determine the antioxidative activities of BECMHFs *in vitro*. Biologically, changes in the reactive oxygen species (ROSs), nitric oxide (NO), and inducible nitric oxide synthase (iNOS) levels after BECMHF treatment were also determined to clarify the possible antioxidative mechanism of BECMHFs.

DPPH radical scavenging activity assay The radical scavenging activity of the BECMHF solution against stable DPPH was determined using a slightly modified DPPH radical scavenging assay (26). Briefly, a 25-μl BECMHF solution was diluted to 4 ml by using methanol and then mixed with 0.5 ml of a freshly prepared 1-mM DPPH solution (Sigma Chemical Co., St. Louis, MO, USA). After 30 min of incubation, the absorbance at 517 nm was measured using a spectrophotometer (U-2001, Hitachi Co., Tokyo, Japan). The DPPH radical scavenging activity was calculated using the following equation (27):

$$\text{DPPH radical scavenging ability } (\%) = [1 - (OD_{517} \text{ of sample with BECMHF solution})/(OD_{517} \text{ of control without BECMHF solution})] \times 100 \quad (2)$$

Ascorbic acid (Sigma Chemical Co.) was used as a positive control.

Superoxide scavenging activity assay The superoxide radical (O_2^-) generated by the xanthine/xanthine oxidase system was measured spectrophotometrically by detecting the product of nitroblue tetrazolium (NBT). The ability of the sample to eliminate superoxide radicals is thus proportional to the reduction in absorbance (28). The 8 BECMHF solutions were separately mixed with 0.15 mM of NADH and 0.74 mM of NBT in sequence and then added promptly to 0.05 mM of PMS to obtain a reduced form of NBT. The amount of NBT in the reduction state was determined through absorbance at a wavelength of 560 nm (28). The superoxide scavenging activities of various BECMHF solutions were measured using the following equation (29):

$$\text{Scavenging activity (SA, \%)} = [1 - (OD_{560} \text{ of sample with BECMHF solution})/(OD_{560} \text{ of control without BECMHF solution})] \times 100 \quad (3)$$

Superoxide dismutase-like activity assay The SOD-like activities of the 8 BECMHF solutions were measured using a light-induced NBT/riboflavin assay (A_{560}) (28). One unit of SOD activity was defined as the number of enzymes that inhibited 50% of the NBT reduction (28). Acetone powder was prepared for each sample with 10 times (w/w) the mass of acetone and dissolved in a potassium phosphate buffer (50 mM, pH 7.8) at a ratio of 1:5 (w/v) for 12 h at 4°C. After centrifuging (4°C, 8000 $\times g$, 30 min), ammonium sulfate was added to yield a 50%–80% saturation at 4°C. Precipitated protein was collected and dissolved in a